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SPECTROSCOPIC CHARACTERISATION OF ORDER–DISORDER TRANSITIONS FOR EXTRACELLULAR POLYSACCHARIDES OF *Arthrobacter* SPECIES*

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ABSTRACT

The conformational behaviour of the extracellular polysaccharides from *Arthrobacter* species of soil-borne bacteria has been investigated by nuclear magnetic resonance relaxation and optical rotation. Polysaccharides from *A. stabilis*, *A. viscosus*, and *A. viscosus* sp. n, in solution at room temperature, all show evidence of an ordered conformation which can be melted out on heating. The temperature course of this transition, however, shows considerable variation with bacterial species. Thus *A. stabilis* polysaccharide shows a very sharp conformational transition centred around 60°, whereas the transitions of the polysaccharides from both strains of *A. viscosus* occur over a much broader temperature-range. The transition for the polysaccharide of *A. viscosus* sp. n is again centred close to 60°, whereas, for *A. viscosus*, melting of the tertiary structure of the polysaccharide is incomplete at 100°. *O*-Deacetylation destroys the ordered conformation of both *A. viscosus* polysaccharides. The ordered structure of *A. stabilis* polysaccharide, by contrast, is stabilised by removal of acyl substituents (which here include succinic half-ester). Understanding of the conformational state of these materials affords considerable insight into their gelation behaviour and unusual solution rheology. The known solution interactions with certain plant polysaccharides suggest a possible biological role for *Arthrobacter* polysaccharides in relationships with components of plant root-systems.

INTRODUCTION

Since the initial production of xanthan by Dr. Jeanes and her group at Peoria, the unusual and industrially attractive solution properties of xanthan gum, the extracellular bacterial polysaccharide of *Xanthomonas campestris*, have attracted considerable scientific and technological interest[1], and it is now established[2] as a commercially viable, industrial hydrocolloid. Deviations from normal polyelectrolyte behaviour have been traced[3,4] to a rigid, ordered, molecular conformation in solution,

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.

which melts out only under conditions of elevated temperature and low ionic strength This understanding provides[5] a unifying interpretation of most of the technologically valuable rheological peculiarities of xanthan[6]. Thus, maintenance of solution viscosity in the presence of high concentrations of salt is a direct consequence of a fixed conformation which, unlike normal random-coil polyanions, cannot alter its molecular dimensions in response to changes in ionic strength, while the extreme shear-thinning behaviour of xanthan solutions is entirely consistent with the ability of rod-like molecules to align in the direction of flow.

Analogous rheological behaviour has been observed[7–9] for extracellular bacterial polysaccharides of *Arthrobacter* species, suggesting the possibility of a similar molecular origin. In an earlier investigation[10] of possible viscous interactions with plant galactomannans, we found evidence of conformational rigidity of *Arhtrobacter* polysaccharides in solution and gels. In the present work, we now characterise the temperature course of order–disorder behaviour, using n.m.r. and optical rotation techniques, which have proved very effective in previous studies[3,4,10–15] of co-operative order in polysaccharide solutions and gels.

SPECTROSCOPIC TECHNIQUES

Single-wavelength optical rotation is now well established as a sensitive and direct probe of polysaccharide conformation. Indeed, a simple quantitative relationship has been demonstrated[16] between changes in the glycosidic angles between adjacent residues and consequent changes in optical activity at the D line. Recent vacuum-ultraviolet, circular-dichroism studies[17] suggest that these chiroptical effects have their origin in two optically active absorption-bands centred around 180 and 160 nm. Discontinuities in the temperature dependence of optical rotation provide a particularly convenient index of co-operative conformation change, and the power and scope of this approach have been demonstrated in elucidation of the thermally induced order–disorder transitions in xanthan[3–5,18] and in the carrageenan[11–13] and agar[14,15] families of algal polysaccharides.

Polymer conformation may also be monitored[11,19,20] by n.m.r. relaxation studies. The decay of induced magnetisation may occur by loss of phase of individual precessing nuclei, and is characterised by the spin–spin relaxation time T_2. As thermal motions interfere with this process, the relaxation rate is inversely related to the degree of molecular mobility. Relaxation behaviour can be measured directly as the time constant for exponential decay of magnetisation, or indirectly from high-resolution linewidth, $\Delta\nu_{\frac{1}{2}}$, from the relationship $T_2 = 1/\pi\Delta\nu_{\frac{1}{2}}$. Thus small molecules moving freely in solution show sharp, narrow, spectral lines, whereas linewidths for solids are so great that no high-resolution spectrum can be detected: the proton T_2 for water is of the order of 1 sec, whereas those for mono- and oligo-saccharides in solution are of the order of hundreds of milliseconds. Typical random-coil polysaccharides in solution show T_2 values around 50 msec, corresponding to high-resolution peaks which, although broader than for monosaccharides, are still clearly discernable. By constrast, such rigid conformations as the double helix of carrageenan

show T_2 values around 50 μsec even in solution, and the corresponding linewidth is so great that all peaks are flattened into the baseline of the high-resolution spectrum.

Arthrobacter POLYSACCHARIDES

In this work, we have studied three extracellular polysaccharides of the genus *Arthrobacter*, cultured by Dr. Jeanes and her colleagues in the U.S. Department of Agriculture Northern Regional Research Laboratory in Peoria. The full primary structures have yet to be established, but their composition is known, and is shown in Table I. The *Arthrobacter viscosus* polysaccharide has been more fully characterised[21] than either of the other samples, and is believed to be based on a linear repeating-unit of:

→4)-β-D-Man*A*p-(1→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→ .

The acetate content corresponds to acetylation of four of the eight hydroxyl groups of the repeating structure. The positions of *O*-acetylation have not yet been established, but this is the subject of a current study by Prof. B. Lindberg and his group in Stockholm. X-Ray diffraction analysis has also been initiated to establish the conformation in the condensed phase, and preliminary results[22] suggest a three-fold helix with a pitch of 4.25 nm. All three polysaccharides show thermally reversible gelation-behaviour at concentrations $> \sim 1\%$ (w/v) in water, and at lower concentrations in the presence of salt.

TABLE I

COMPOSITION OF *Arthrobacter* EXTRACELLULAR POLYSACCHARIDES

Synthesising bacterium	*NRRL strain*	*Constituent sugars and molar ratios*	*Substituents* (%)		*Refs.*
A. stabilis	B 3225	Glc:Gal 2:1	*O*-Acetyl	5.0	30
			O-Succinoyl	3–5	
			Pyruvate	5.1	
A. viscosus	B 1973	Glc:Gal:ManA 1:1:1	*O*-Acetyl	25	21, 29
A. viscosus sp. n.	B 1797	Glc:Gal:GlcA 3:1:1	*O*-Acetyl	8.0	30
			Pyruvate	5.5	

EXPERIMENTAL

Materials. — The *Arthrobacter* polysaccharide samples, including *O*-deacylated materials, were generously donated by Dr. Allene Jeanes. These materials were prepared and isolated in the potassium salt form as previously reported[21], and were used without further purification. All solutions were brought to neutrality prior to physical measurements. Samples for n.m.r. studies were deuterated by repeated (3×) lyophilisation from D_2O.

Methods. — High-resolution ^1H-n.m.r. spectra were recorded at 100 MHz in D_2O solution (0.5% w/v) with a Varian XL-100 Fourier-transform spectrometer, by

using 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) as chemical-shift reference. Integrated peak areas were referred to an external standard of pyrazine in D_2O doped with $MnCl_2$, contained in a 1-mm capillary tube located concentrically within the sample tube. Optical rotation measurements were made at 365 nm on a Perkin–Elmer 241 polarimeter, with a 10-cm, thermostatted cell and a sample concentration of ~0.5% (w/v). The temperature was controlled by a Haake circulating water-bath.

RESULTS

Fig. 1 shows the high-resolution ^{1}H-n.m.r. spectrum of three *Arthrobacter* polysaccharides in both their unmodified and *O*-deacylated form, at 95°. The polysaccharide from *A. viscosus* shows a large upfield resonance at δ 2.14, assigned to *O*-acetyl groups, and a very much smaller and sharper peak at δ 1.87, which is attributed to free acetate, presumably arising from limited saponification during storage. These two singlet resonances are also observed for the polysaccharide of *A. viscosus* sp. n, in addition to a third, upfield singlet at δ 1.44, which is attributed to

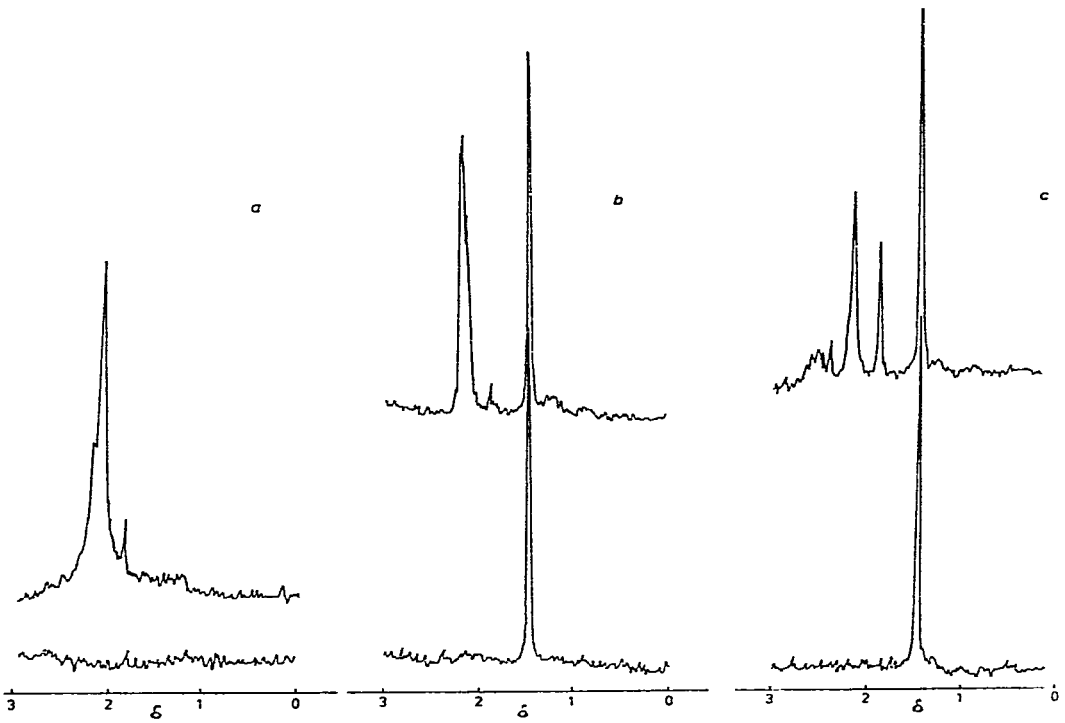

Fig. 1. High-resolution n.m.r. spectra of extracellular polysaccharides from (*a*) *A. viscosus*, (*b*) *A. viscosus* sp. n and (*c*) *A. stabilis*. In each example, the upper spectrum is for the unmodified polysaccharide, and the lower spectrum for the corresponding *O*-deacylated material. All spectra were recorded at 95°.

pyruvate acetal. Chemical-shift values are in excellent agreement with those obtained previously for xanthan[4]. The extracellular polysaccharide of *A. stabilis* also shows well resolved, singlet resonances from pyruvate acetal, *O*-acetyl, and a limited amount of free acetate, together with a complex resonance around δ 2.5 which, on the basis of the known chemical composition (Table I), is assigned to *O*-succinoyl substituents.

The high-resolution spectrum of *A. stabilis* polysaccharide collapses completely on cooling, indicating conversion into a rigid conformation. This process is fully reversible, and its temperature-course is identical for all three substituents, as shown

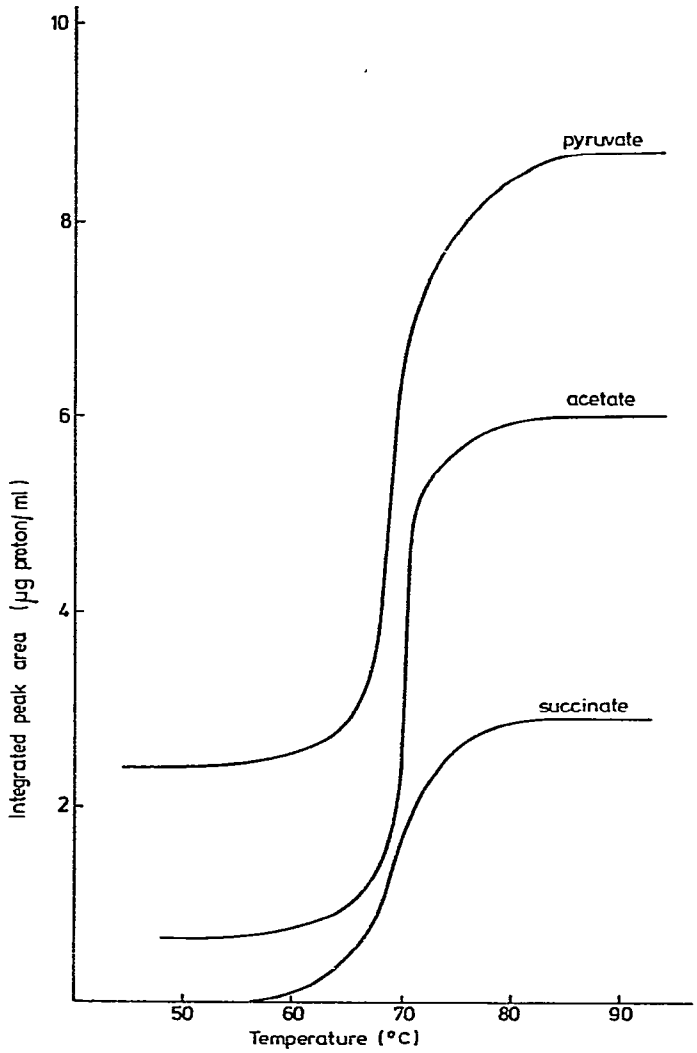

Fig. 2. Temperature course of the collapse of the high-resolution ^{1}H-n.m.r. spectrum of *A. stabilis* polysaccharide, on cooling. Polysaccharide concentration = 0.5% (w/v); no added salt.

in Fig. 2. The sigmoidal nature of the n.m.r. temperature-profile is indicative of a cooperative order–disorder transition. The disappearance of the high-resolution spectrum, even at non-gelling concentrations, suggests an origin in molecular change, rather than in bulk-gel properties. This interpretation is confirmed by optical-rotation measurements which show a similar sharp discontinuity in temperature dependence. As shown in Fig. 3, the order–disorder transition is shifted to higher temperature on addition of salt, consistent with stabilisation of the ordered structure by decrease of internal electrostatic repulsions through charge screening.

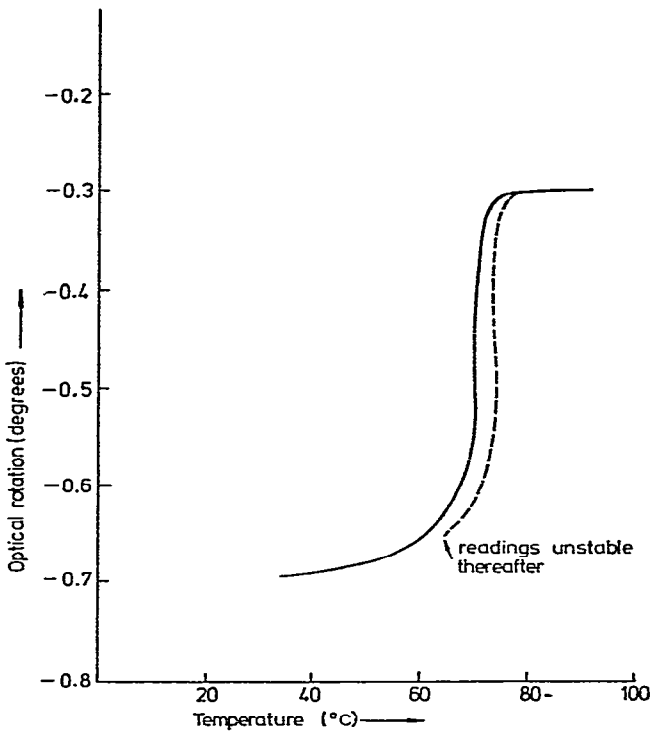

Fig. 3. Ionic-strength dependence of the order–disorder transition of *A. stabilis* polysaccharide, as monitored by optical rotation. Pathlength, 10 cm; 1% (w/v) in distilled water (———), and in 0.1M KCl (– – – –).

The extracellular polysaccharides from *A. viscosus* and *A. viscosus* sp. n show similar indications of order-disorder behaviour in solution, as monitored by optical rotation (Fig. 4) and ^{1}H n.m.r. (Figs. 5 and 6). The transition midpoint for the polysaccharide of *A. viscosus* sp. n is close to that for *A. stabilis*, although the temperature course of the transition is considerably broader. The order–disorder transition of the polysaccharide from *A. viscosus* is even broader, and centred about 40° higher, so that denaturation is incomplete at 100°. *O*-Deacylated samples of *A. viscosus* and *A. viscosus* sp. n polysaccharides show no evidence of conformational

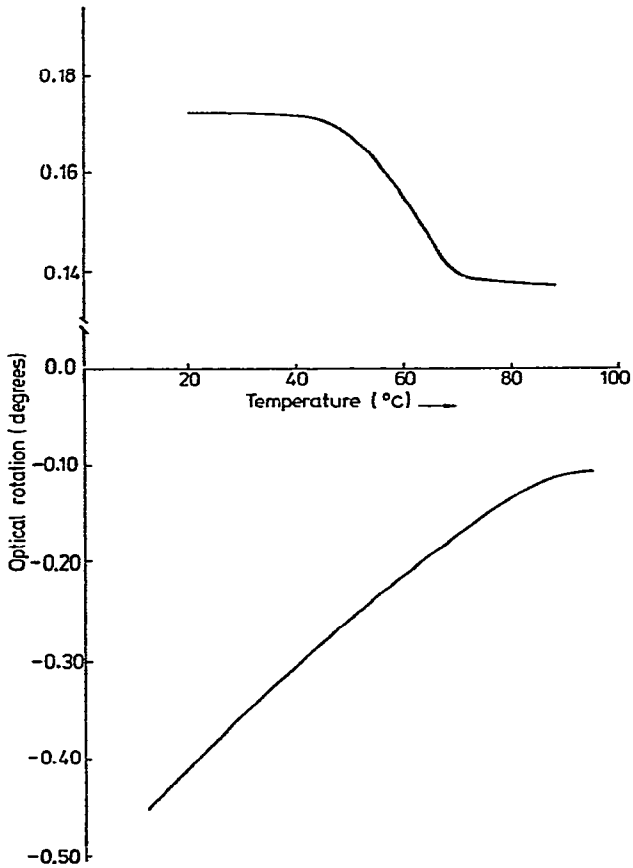

Fig. 4. Optical rotation–temperature profile for the extracellular polysaccharides from *A. viscosus* (lower curve), and *A. viscosus* sp. n (upper curve). Pathlength, 10 cm; 365 nm; 1% (w/v); no added salt.

order, as at room temperature both display high-resolution ^{1}H-n.m.r. spectra typical of a polysaccharide random-coil. *O*-Deacylated *A. stabilis* polysaccharide, by contrast, shows an order–disorder transition having a similar amplitude of optical-rotation change[10], and similar temperature-induced changes in n.m.r. behaviour (Fig. 7), to those of the unmodified material.

DISCUSSION

The usual consequence of addition of salt to polyelectrolyte solution is a decrease in viscosity, as intramolecular, electrostatic repulsions are decreased by charge screening, and the polymer coil is allowed to collapse[2,3]. By contrast, the viscosity of aqueous solutions of the *Arthrobacter* polysaccharides is enhanced on increasing the ionic strength[7,8]. This unusual, but industrially attractive, behaviour (also displayed

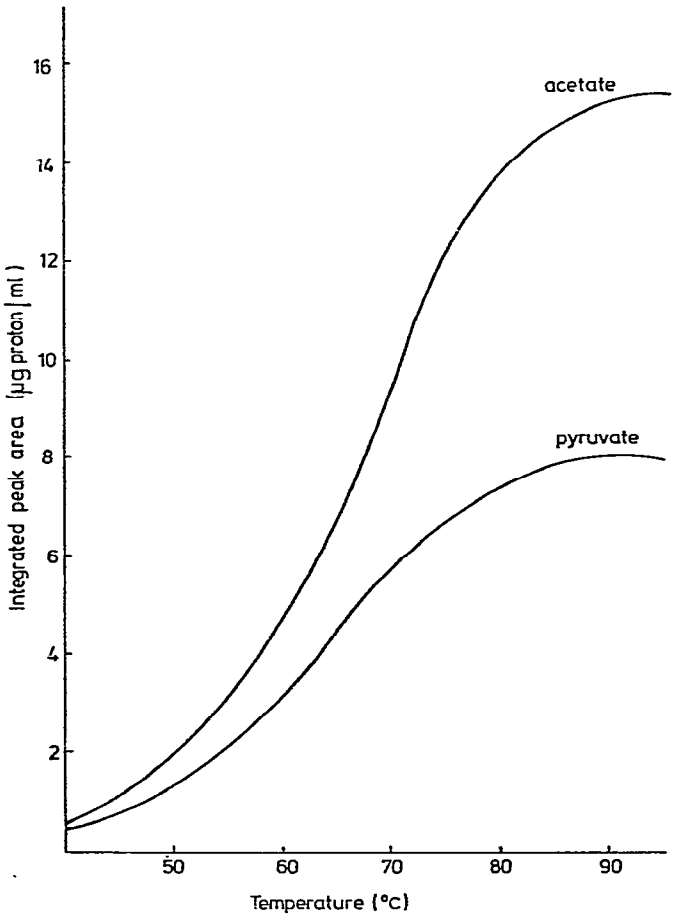

Fig. 5. Temperature course of the collapse of the high-resolution ^{1}H-n.m.r. spectrum of *A. viscosus* sp. n polysaccharide on cooling. Polysaccharide concentration=0.5% (w/v); no aadded salt.

by xanthan)[6], may be readily explained in terms of decrease in electrostatic repulsion between extended, conformationally rigid, molecular species, with consequent enhancement of intermolecular association and aggregation. Further evidence of intermolecular association comes from solution rheology. Polysaccharide solutions normally show continuous deformation under stress, whereas solutions of the ordered bacterial polysaccharides appear to show a yield point[9]. Thus, at high shear they flow like normal polymer solutions, but under low-shear conditions they behave essentially as gels and hence show excellent particle suspension and emulsion-stabilisation properties.

As Flory has pointed out[24], the aggregation of random-coil polymers must involve loss of conformational entropy, which must therefore be offset by favourable energy terms. Aggregation of rigid, rod-like molecules, by contrast, does not involve

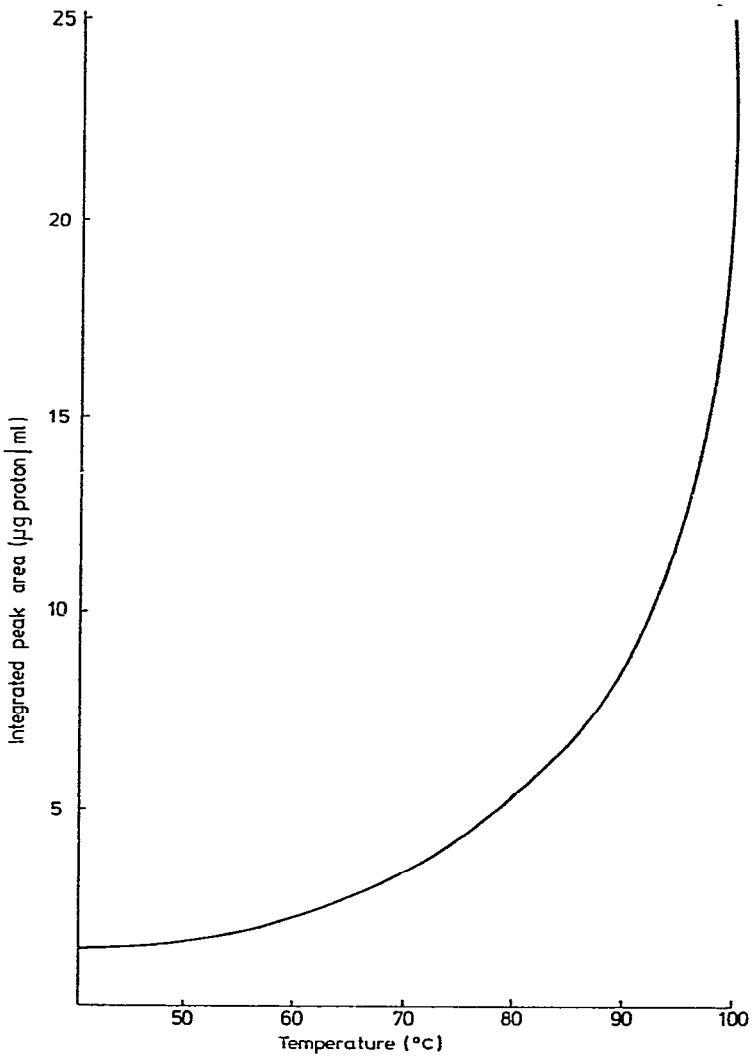

Fig. 6. Temperature course of the collapse of the high-resolution ^{1}H-n.m.r. spectrum of *A. viscosus* polysaccharide on cooling (as monitored by the *O*-acetyl resonance upfield). Polysaccharide concentration = 0.5% (w/v); no added salt.

this conformational-entropy loss, and can therefore occur when intermolecular attraction is smaller. We believe that this accounts for the extensive, but tenuous, intermolecular association between ordered polysaccharides in solution, which is indicated by the rheological evidence. The striking parallels in rheological behaviour between *Arthrobacter* exopolysaccharides and xanthan can therefore be traced to analogous tertiary structures. Future attempts to develop technologically valuable bacterial polysaccharide systems might profitably take account of this importance of

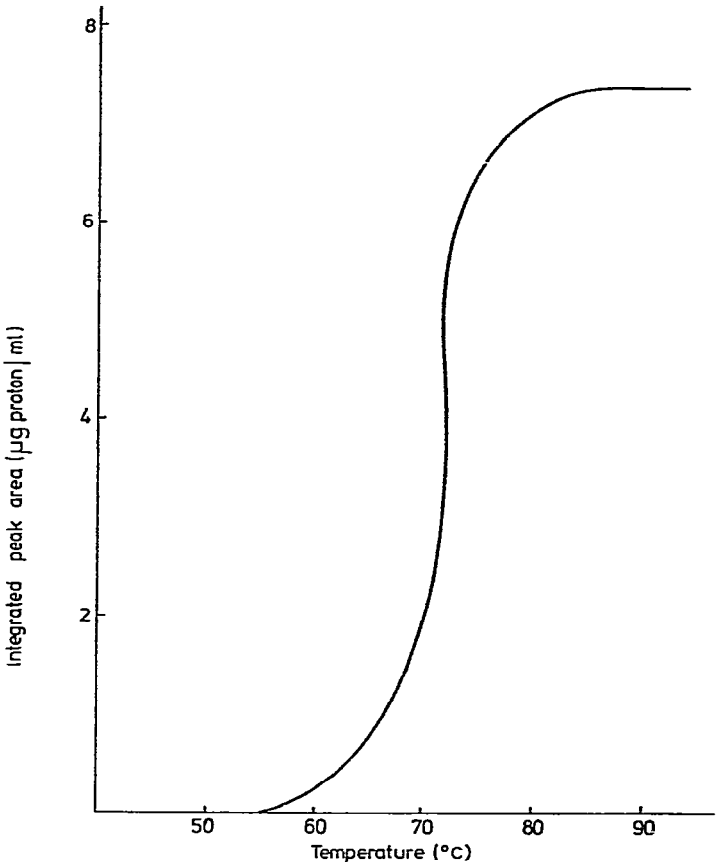

Fig. 7. Temperature course of the collapse on cooling of the high-resolution ^{1}H-n.m.r. spectrum for the *O*-deacylated extracellular polysaccharide from *A. stabilis* (as monitored by the pyruvate acetal resonance upfield). Polysaccharide concentration=0.5% (w/v); no added salt.

conformation, in addition to such more-obvious factors as efficiency of conversion and ease of recovery.

Recent observations of ageing effects in *Arthrobacter* polysaccharide samples[25] suggest a time-dependent aggregation process analogous to those demonstrated[10] for certain plant polysaccharide systems. This could explain the apparent discrepancy between previous reports of viscous interactions of *Arthrobacter* polysaccharides with galactomannans and the results of our recent investigation[10] which showed no such effects, as our experimental conditions were designed to minimise self aggregation, whereas current observations[25] suggest that synergistic interaction is greatest for extensively aggregated *Arthrobacter* polysaccharide samples.

Studies of the distribution of soil microflora show that the bacterial population within the domain of the plant root-system (the rhizosphere) differs both quantitatively and qualitatively from that in the surrounding soil[26]. Microscopic examina-

tion[27] of plant root surfaces reveals a bacterial layer several cells deep, between the root and the soil. Classification of the Gram-negative bacteria which predominate in the rhizosphere presents some difficulty, but studies of rhizosphere isolates from grass and clover[28] suggest a prevalence of *Arthrobacter* species, although smaller populations of other genera were also identified. The origin of bacterial recognition and attachment to plant root surfaces has yet to be elucidated. Evidence of specific interactions between plant polysaccharides and the extracellular polysaccharides of *Arthrobacter* species suggests that polysaccharide–polysaccharide interactions could be involved, possibly analogous in some respects to those proposed[4] in host–pathogen recognition involving *Xanthomonas* species.

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